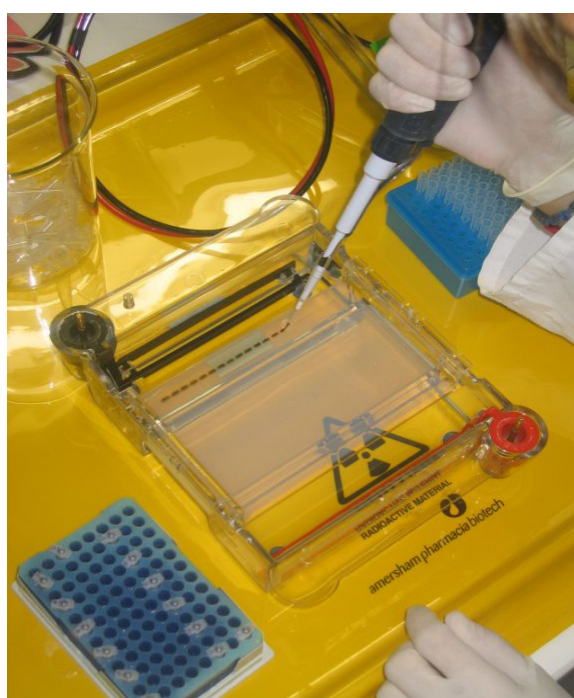


# Report on the Verification of the Performance of Bt11, MIR604 and GA21 Maize Event-specific Methods on the Maize Event Bt11 x MIR604 x GA21 Using Real-time PCR

Validation Report and Protocols

C. Charles-Delobel, B. Munaro, M. Mazzara, G. Van den Eede



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# **Report on the Verification of the Performance of Bt11, MIR604 and GA21 Event-specific Methods on the Maize Event Bt11 x MIR604 x GA21 Using Real-Time PCR**

17 May 2010

**Joint Research Centre  
Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit**

## **Executive Summary**

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of three quantitative event-specific methods on the maize event Bt11 x MIR604 x GA21 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) which combines the Bt11, MIR604 and GA21 transformation events. The three methods have been validated individually on single-trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines <sup>(1, 2)</sup>.


In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Syngenta Seeds S.A.S. provided the detection methods and the control samples: genomic DNA extracted from homogenised seeds of Bt11 x MIR604 x GA21 maize (NP2673GA21xNP2171Bt11+MIR604), genomic DNA extracted from homogenised seeds of non-GM maize (NP2673/NP2171) and flour ground from seeds of NP2673GA21xNP2171Bt11+MIR604 and from seeds of NP2673/NP2171. The EURL-GMFF prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to the European Network of GMO Laboratories (ENGL) method performance requirements (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this EURL-GMFF in-house verification study are made publicly available at <http://gmo-crl.jrc.ec.europa.eu/>

*Drafted by:*

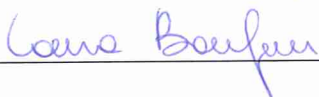
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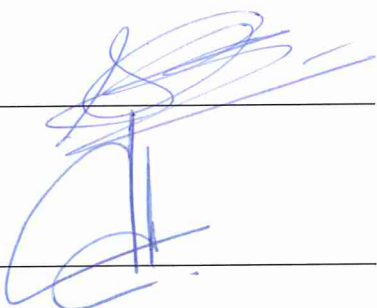
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## Report on Steps 1-3 of the Validation Process

Syngenta Seeds S.A.S. submitted the detection methods and control samples of the maize event Bt11 x MIR604 x GA21 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EURL-GMFF Validation Process", <http://gmo-crl.jrc.ec.europa.eu/doc/Description%20CRL%20validation%20process.pdf>).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" ([http://gmo-crl.jrc.ec.europa.eu/doc/Min\\_Perf\\_Requir\\_Analyt\\_methods\\_131008.pdf](http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requir_Analyt_methods_131008.pdf)) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). The scientific assessment of the detection methods for the Bt11 x MIR604 x GA21 was positively concluded in May 2008.

The event-specific detection methods for the three single Bt11, MIR604 and GA21 maize events were validated by the EURL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). Hence, the detection methods applied on the maize event Bt11 x MIR604 x GA21 did not undergo a full validation process. The EURL-GMFF performed a verification of the detection methods to verify that they exhibit a comparable performance on samples of event Bt11 x MIR604 x GA21 combining the three traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In June 2008, the EURL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.09%-8%, 0.1%-6% and 0.09%-8% for Bt11, MIR604 and GA21 respectively, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were mostly within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the EURL-GMFF (step 3) is available on request.

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# 1. Introduction

Syngenta Seeds S.A.S. submitted the detection methods for Bt11, MIR604 and GA21 and the control samples of the maize event Bt11 x MIR604 x GA21 (unique identifier SYN-BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out a verification of the three event-specific methods for the detection and quantification of Bt11, MIR604 and GA21 in the Bt11 x MIR604 x GA21 maize event combining the three traits. The single methods had been previously validated by international collaborative studies on the single-trait maize events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

Upon reception of methods, samples and related data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the verification of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following EURL-GMFF operational procedures. The EURL-GMFF method verification was concluded in June 2008.

A method submitted by the applicant for DNA extraction from maize seeds was evaluated by the EURL-GMFF in order to confirm its performance characteristics. The protocol for DNA extraction is available at <http://gmo-crl.jrc.ec.europa.eu/>.

The procedure of verification consisted of a quantitative real-time Polymerase Chain Reaction (PCR). The methodology consists of three event-specific real-time quantitative TaqMan<sup>®</sup> PCR procedures for the determination of the relative content of events Bt11, MIR604 and GA21 DNA to total maize DNA in the Bt11 x MIR604 x GA21 maize event. The procedures are simplex systems, in which the events Bt11, MIR604 and GA21 are quantified in reference to the maize *adh1* (alcohol dehydrogenase-1) taxon-specific endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ✓ ISO 5725:1994 <sup>(1)</sup>
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" <sup>(2)</sup>.

## 2. Materials

The following control samples were provided by the applicant:

- genomic DNA extracted from homogenised seeds of Bt11 x MIR604 x GA21 maize (NP2673GA21xNP2171Bt11+MIR604),
- genomic DNA extracted from homogenised seeds of non-GM maize (NP2673/NP2171),

in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of Bt11 x MIR604 x GA21 and non-GM maize genomic DNA at different GMO contents were prepared in a constant amount of total maize DNA.

The validated methods for the individual Bt11, MIR604 and GA21 events were applied in the verification as published and available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

In tables 1 are reported the five GM contents used in the verification of the Bt11, MIR604 and GA21 methods.

Table 1. Bt11, MIR604 and GA21 GM contents in maize event Bt11 x MIR604 x GA21.

<b>Bt11 GM %</b> (GM DNA / Non-GM DNA *100)	<b>MIR604 GM %</b> (GM DNA / Non-GM DNA *100)	<b>GA21 GM %</b> (GM DNA / Non-GM DNA *100)
0.09	0.10	0.09
0.40	0.40	0.50
0.90	0.90	0.90
5.00	2.50	5.00
8.00	6.00	8.00

## 3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the target taxon-specific assay (*adh1*). Five GM contents per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (Bt11, MIR604 and GA21), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level.



## 4. Method

To detect Bt11, MIR604 and GA21 events in maize event Bt11 x MIR604 x GA21, three specific fragments of 68-bp, 76-bp and 101-bp respectively, corresponding to the integration regions of the constructs into the plant genome, were amplified using specific primers.

For relative quantification of events Bt11, MIR604 and GA21 DNA, was employed a maize-specific target taxon system amplifying a 135-bp fragment of the maize gene *adh1* (alcohol dehydrogenase 1), using *adh1* specific primers and an *adh1* specific probe labelled with VIC and TAMRA.

Standard curves were generated for each GM specific system (Bt11, MIR604 or GA21), by plotting  $\Delta C_t$  values of the calibration samples against the logarithm of the amount of events Bt11, MIR604 or GA21 DNA, and by fitting a linear regression into these data. Thereafter, the relative amount of event Bt11, MIR604 or GA21 is estimated by means of the regression function from the normalised  $\Delta C_t$  values of the unknown samples.

Detailed information on standard curve samples preparation is available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

## 5. Deviations reported

The Sigma JumpStart Taq Ready Mix was supplemented with 600 nM sulforhodamine for all PCR reactions (Bt11, MIR604, GA21 and Adh1 specific assays), i.e. the final concentration of sulforhodamine in each PCR reaction was 300 nM.

## 6. Summary of results

### *PCR efficiency and $R^2$*

The values of the slopes of the standard curves, the PCR efficiency and the  $R^2$  (expressing the linearity of the regression) are presented for Bt11, MIR604 and GA21 methods in tables 2, 3 and 4. The data for the eight runs for each method are reported. The PCR efficiency was calculated using the formula  $[10^{(-1/\text{slope})}-1]*100$ .

Table 2. Values of standard curve slope, PCR efficiency and  $R^2$  of the Bt11 method (on event Bt11 x MIR604 x GA21).

Run	Bt11		
	Slope	PCR Efficiency (%)	$R^2$
1	-3.12	109	1.00
2	-3.46	94	1.00
3	-3.22	104	1.00
4	-3.49	93	1.00
5	-3.25	103	1.00
6	-3.33	100	1.00
7	-3.24	104	1.00
8	-3.27	102	1.00
Mean	-3.30	101	1.00

Table 3. Values of standard curve slope, PCR efficiency and  $R^2$  of the MIR604 method (on event Bt11 x MIR604 x GA21).

Run	MIR604		
	Slope	PCR Efficiency (%)	$R^2$
1	-3.40	97	1.00
2	-3.26	103	1.00
3	-3.41	97	1.00
4	-3.45	95	1.00
5	-3.29	101	1.00
6	-3.36	99	1.00
7	-3.40	97	1.00
8	-3.24	103	1.00
Mean	-3.35	99	1.00

Table 4. Values of standard curve slope, PCR efficiency and  $R^2$  of the GA21 method (on event Bt11 x MIR604 x GA21).

Run	GA21		
	Slope	PCR Efficiency (%)	$R^2$
1	-3.15	108	1.00
2	-3.12	109	1.00
3	-3.37	98	1.00
4	-3.26	103	1.00
5	-3.13	109	1.00
6	-3.25	103	1.00
7	-3.35	99	1.00
8	-3.17	107	1.00
Mean	-3.22	104	1.00

The mean PCR efficiencies for the Bt11, MIR604 and GA21 methods were 101%, 99% and 104%, respectively. The  $R^2$  was 1.00 for all methods. Overall, the data reported in Tables 2, 3 and 4 confirmed the appropriate performance characteristics of the three methods tested on Bt11 x MIR604 x GA21 maize samples in terms of PCR efficiency and linearity.

## 7. Method performance requirements

The results of the verification study for the Bt11, MIR604 and GA21 detection methods applied to event Bt11 x MIR604 x GA21 maize DNA are reported in tables 5, 6 and 7, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the EURL-GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), see also Annex 1). In addition, tables 5, 6 and 7 report estimates of the trueness and relative repeatability standard deviation for each GM level for the three methods.

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSDr %) of the Bt11 method on event Bt11 x MIR604 x GA21 maize DNA.

Bt11					
Unknown sample GM%	Expected value (GMO %)				
	0.09	0.4	0.9	5.0	8.0
Mean	0.09	0.39	0.92	5.44	9.01
SD	0.02	0.05	0.06	0.44	1.08
RSDr (%)	20	13	7	8	12
Bias (%)	-1.5	-2.0	1.8	8.9	13

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MIR604 method on event Bt11 x MIR604 x GA21 maize DNA.

MIR604					
Unknown sample GM%	Expected value (GMO %)				
	0.1	0.4	0.9	2.5	6.0
Mean	0.10	0.41	0.95	2.54	6.43
SD	0.02	0.06	0.09	0.20	0.64
RSDr (%)	15	15	9	8	10
Bias (%)	3.6	3.5	5.8	4.7	7

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the GA21 method on event Bt11 x MIR604 x GA21 maize DNA.

GA21					
Unknown sample GM%	Expected value (GMO %)				
	0.09	0.5	0.9	5.0	8.0
Mean	0.09	0.48	0.92	5.51	8.77
SD	0.01	0.04	0.06	0.50	0.79
RSDr (%)	9	9	7	9	9
Bias (%)	-1.5	-3.0	2.8	10	10

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be  $\pm 25\%$  across the entire dynamic range. As shown in tables 5, 6 and 7, all methods satisfied the requirement throughout their respective dynamic ranges; in fact, the highest bias was 13%, 7% and 10% for the Bt11, MIR604 and GA21 methods, respectively.

Tables 5, 6 and 7 further document the *relative repeatability standard deviation* (RSD<sub>r</sub> %) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the EURL-GMFF requires that RSD<sub>r</sub> values are below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>]). As it can be observed from the values reported in Tables 5, 6 and 7, the three methods satisfied this requirement throughout their respective dynamic ranges; in fact, the highest RSD<sub>r</sub> were 20%, 15% and 9% for the Bt11, MIR604 and GA21 methods, respectively.

## 8. Comparison of method performance between event Bt11 x MIR604 x GA21 and the single trait events

An indicative comparison of the three method performances on the maize event Bt11 x MIR604 x GA21 and on the single trait events is shown in tables 8, 9 and 10. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 8. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the Bt11 detection method on event Bt11 x MIR604 x GA21 and on event Bt11.

Trueness and repeatability of Bt11 quantification on Bt11 x MIR604 x GA21			Trueness and repeatability of Bt11 quantification on single event Bt11 *		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.09	-1.5	20	0.09	2.2	17
0.4	-2.0	13	0.4	-1.9	13
0.9	1.8	7	0.9	1.8	11
5.0	8.9	8	5.0	-5.2	13
8.0	13	12	8.0	-1.2	9

\*method validated in collaborative trial (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 9. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the MIR604 detection method on event Bt11 x MIR604 x GA21 and on event MIR604.

Trueness and repeatability of MIR604 quantification on Bt11 x MIR604 x GA21			Trueness and repeatability of MIR604 quantification on single event MIR604 *		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.1	3.6	15	0.1	3.6	24
0.4	3.5	15	0.4	3.1	17
0.9	5.8	9	0.9	-1.0	12
2.5	4.7	8	2.5	0.7	16
6.0	7	10	6.0	-3.6	14

\*method validated in collaborative trial (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 10. Trueness (bias %) and relative repeatability standard deviation (RSDr %) of the GA21 detection method on event Bt11 x MIR604 x GA21 and on event GA21.

Trueness and repeatability of GA21 quantification on Bt11 x MIR604 x GA21			Trueness and repeatability of GA21 quantification on single event GA21 *		
GM%	Bias (%)	RSDr (%)	GM%	Bias (%)	RSDr (%)
0.09	-1.5	9	0.09	-8.7	23
0.5	-3.0	9	0.5	0.8	17
0.9	2.8	7	0.9	1.6	20
5.0	10	9	5.0	-5.6	20
8.0	10	9	8.0	-8.5	17

\*method validated in collaborative trial (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

The Bt11 method (table 8) showed a lower trueness value on the stacked event at the 8% GM content (13% vs 1.2%) and a value similar but positive at the 5% GM content (8.9% vs -5.2%).

The MIR604 method (table 9) showed a higher bias on the stacked event at the 0.9% (5.8% vs -1.0%), 2.5% (4.7% vs 0.7%) and 6% GM contents (7% vs -3.6%).

The GA21 method (table 10) showed lower bias on the stacked event at the 0.09% GM content (-1.5% vs -8.7%), a higher bias at 5% GM (10% vs -5.6%) and similar bias but of opposite strength (positive instead of negative) at the 8% GM content (10% vs -8.5%).

For all method the trueness was within the acceptance value of  $\pm 25\%$  as set by ENGL.

For relative repeatability standard deviation (RSDr %), the Bt11 and MIR604 methods showed similar values when applied to event Bt11 x MIR604 x GA21, compared to the single events, with the exception of the MIR604 method showing lower RSDr % at 0.1% and 2.5% GM levels; the GA21 method showed a lower RSD % for all GM contents of the range. In all cases, the results were below the ENGL acceptance level established at maximum 25%.

Therefore, the Bt11, MIR604 and GA21 detection methods developed to detect and quantify the single events could be equally applied for the quantification of the respective events combined in event Bt11 x MIR604 x GA21, as demonstrated by the verification study.

## 9. Conclusions

The overall method performance of the three event-specific methods for the quantitative detection of events Bt11, MIR604 and GA21 combined in maize event Bt11 x MIR604 x GA21 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

The results obtained during the verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

## 10. Quality assurance

The EURL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32232) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

## 11. References

1. Horwitz, W., 1995, Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.
2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

## 12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

### **Method Acceptance Criteria**

#### ***Applicability***

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

#### ***Practicability***

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

#### ***Specificity***

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

#### ***Dynamic Range***

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative



requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

### ***Accuracy***

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within  $\pm 25\%$  of the accepted reference value over the whole dynamic range.

### ***Amplification Efficiency***

Definition: The rate of amplification that leads to a theoretical slope of  $-3.32$  with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency =  $[10^{(-1/\text{slope})} - 1]$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of  $(-3.1 \geq \text{slope} \geq -3.6)$

### ***R<sup>2</sup> Coefficient***

Definition: The R<sup>2</sup> coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R<sup>2</sup> should be  $\geq 0.98$ .

### ***Repeatability Standard Deviation (RSD<sub>r</sub>)***

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

*Note:* Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

### ***Limit of Quantitation (LOQ)***

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than  $1/10^{\text{th}}$  of the value of the target concentration with an RSD<sub>r</sub>  $\leq 25\%$ . Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

### ***Limit of Detection (LOD)***

**Definition:** The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

**Acceptance Criterion:** LOD should be less than  $1/20^{\text{th}}$  of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring  $\leq 5\%$  false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

### ***Robustness***

**Definition:** The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

**Acceptance Criterion:** The response of an assay with respect to these small variations should not deviate more than  $\pm 30\%$ . Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

## **Method Performance Requirements**

### ***Dynamic Range***

**Definition:** In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

**Acceptance Criterion:** The dynamic range of the method should include the  $1/10$  and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

### ***Reproducibility Standard Deviation ( $RSD_R$ )***

**Definition:** The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

**Acceptance Criterion:** The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An  $RSD_R < 50\%$  is acceptable for concentrations below 0.2%.

### ***Trueness***

**Definition:** The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

**Acceptance Criterion:** The trueness should be within  $\pm 25\%$  of the accepted reference value over the whole dynamic range.



# **Event-specific Method for the Quantification of Maize Line Bt11 Using Real-time PCR**

## **Protocol**

**20 June 2008**

**Joint Research Centre  
Institute for Health and Consumer Protection  
Biotechnology & GMOs Unit**

### **Method development:**

Syngenta Seeds S.A.S.

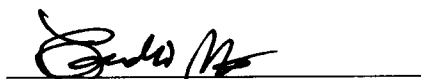
### **Collaborative trial:**

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)  
Biotechnology & GMOs Unit

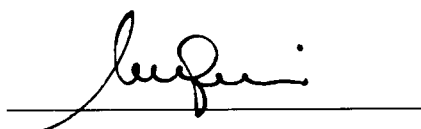
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## 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event Bt11 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in the PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of event Bt11 DNA, a 68-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For the relative quantification of event Bt11 DNA, a maize-specific reference system amplifies a 135-bp fragment of the maize endogenous *alcohol dehydrogenase 1* gene (*adh1*), using two specific primers and an *adh1* gene-specific probe labelled with VIC and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event Bt11 DNA in a test sample, the normalised  $\Delta Ct$  values of the calibration samples are used to calculate by linear regression a reference curve  $\Delta Ct$ -formula. The normalised  $\Delta Ct$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of Bt11 event DNA is estimated.

## 2. Validation status and performance characteristics

### 2.1 General

The method has been optimised for suitable DNA extracted from maize seeds containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative study using DNA samples at different GM% contents.

## 2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in November 2007.

Each participant received twenty unknown samples containing Bt11 maize genomic DNA at five GM% contents, ranging from 0.09% to 8.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM Bt11 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.it/statusofdoss.htm>

## 2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.04% in 250 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

## 2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.08% in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09%.

## 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to Bt11 event and thus imparts event-specificity to the method.

The specificity of the Bt11 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR by the applicant against DNA extracted from samples containing GM maize Bt11, Bt10, NK603, MON810, MON863, MON810 x MON863, TC1507, MIR604, Bt176, GA21, MON88017, T25 and Herculex RW (59122).

According to the applicant, none of the above mentioned GM lines tested, except the positive control Bt11, produced amplification signals in replicated samples when 100 ng total DNA per reaction were used.

### 3. Procedure

#### 3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. "flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- Equipment used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) should be suitable for PCR and molecular biology applications; it should be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4 °C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

#### 3.2 Real-time PCR for quantitative analysis of Bt11 maize

##### 3.2.1 General

The PCR set-up for the taxon specific target sequence (*adh1*) and for the GMO (Bt11) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 250 ng of template DNA per reaction well is recommended.



The method is developed for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

### 3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of Bt11 DNA in a total amount of 250 ng maize DNA. The GM content of the standard samples ranges from 10% to 0.08%.

A calibration curve is produced by plotting the  $\Delta C_t$  values of calibration samples against the logarithm of the respective GM% contents; the slope (a) and the intercept (b) of the calibration curve ( $y = ax + b$ ) are then used to calculate the mean GM% content of the blind samples based on their normalised  $\Delta C_t$  values.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents on ice.**
2. In two reaction tubes (one for Bt11 system and one for the *adh1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *adh1* reference system.

Component	Final concentration	µL/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
Zm <i>adh1</i> – F primer (10 µM)	300 nM	0.75
Zm <i>adh1</i> – R primer (10 µM)	300 nM	0.75
Zm <i>adh1</i> – P probe (10 µM)	200 nM	0.50
Nuclease free water	#	5.50
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the Bt11 specific system.

Component	Final concentration	µL/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
Bt11-ev-f1 primer (10 µM)	200 nM	0.50
Bt11-ev-r5 primer (10 µM)	200 nM	0.50
Bt11-ev-p1 probe (10 µM)	150 nM	0.38
Nuclease free water	#	6.12
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the Bt11 and one for the *adh1* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g.  $20 \times 3 = 60$  µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g.  $5 \times 3 = 15$  µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
- Spin down the tubes in a microcentrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately  $250 \times g$  for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR with cycling conditions described in Table 3

Table 3. Cycling program for maize Bt11/*adh1* systems

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	UNG	50 °C	120	No	1
2	Initial denaturation	95 °C	600	No	1
3	Amplification	Denaturation	95 °C	No	40
		Annealing & Extension	60 °C	Yes	

### 3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. Bt11) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update (or apply)” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at  $Ct = 25 - 3 = 22$ ).
- c) Save the settings.
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *adh1* system).
- e) Save the settings and export all the data into an Excel file for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the Ct-values for each reaction.

The Reference  $\Delta Ct$ -curve is generated by plotting the  $\Delta Ct$ -values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the regression formula is used to estimate the relative amount (%) of Bt11 event in the unknown samples of DNA.

## 4. Materials

### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)

- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 mL reaction tubes

## 4.2 Reagents and solutions

- Sigma JumpStart Taq ReadyMix (2x), Sigma Aldrich Ltd Cat No P-2893
- Sulforhodamine 101, Sigma Cat No S-7635
- 1 M MgCl<sub>2</sub>, Sigma Aldrich Ltd Cat No M-1028

### 10000x Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 mL nuclease free water to make a 1.5 mM stock solution.

Vortex well and store at -20 °C.

### Supplemented 2x Sigma JumpStart ReadyMix:

For 50 mL: to Sigma Jumpstart Taq ReadyMix (2X), add:

- 550 µL of 1 M MgCl<sub>2</sub>
- 20 µL 10000x Sulforhodamine 101.

Vortex well and store at 4 °C for up to 1 year.

## 4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
Bt11 target sequence	
Bt11-ev-f1 primer	5' – TGT GTG GCC ATT TAT CAT CGA -3'
Bt11-ev-r5 primer	5' – CGC TCA GTG GAA CGA AAA CTC -3'
Bt11-ev-p1 probe	FAM 5'- TTC CAT GAC CAA AAT CCC TTA ACG TGA GT -3' TAMRA
Reference gene <i>adh1</i> target sequence	
Zm <i>adh1</i> – F primer	5' – CGT CGT TTC CCA TCT CTT CCT CC-3'
Zm <i>adh1</i> – R primer	5' – CCA CTC CGA GAC CCT CAG TC -3'
Zm <i>adh1</i> - P probe	VIC 5' – AAT CAG GGC TCA TTT TCT CGC TCC TCA-3' TAMRA



# **Event-specific Method for the Quantification of Maize Line MIR604 Using Real-time PCR**

## **Protocol**

**30 March 2010**

**Joint Research Centre  
Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit**

### **Method development:**

Syngenta Seeds S.A.S.

### **Collaborative trial:**

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)  
Molecular Biology and Genomics Unit

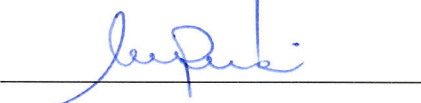
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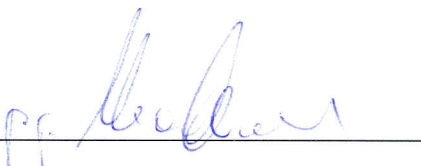
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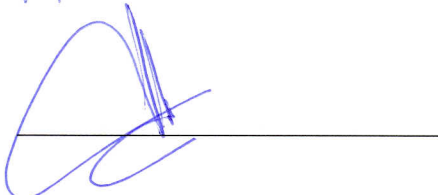
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*Compliance to CRL Quality System*  
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## 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MIR604 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in the PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of event MIR604 DNA, a 76-bp fragment of the recombination region between the insert and the plant genome (located at the 5' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For the relative quantification of event MIR604 DNA, a maize-specific reference system amplifies a 136-bp 135-bp fragment of the maize endogenous *Alcohol dehydrogenase* gene (*Adh1*), using a pair of specific primers and an *Adh1* gene-specific probe labelled with VIC and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MIR604 DNA in a test sample, the normalised  $\Delta C_t$  values of the calibration samples are used to calculate by linear regression a reference curve  $\Delta C_t$ -formula. The normalised  $\Delta C_t$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of MIR604 event DNA is estimated.

## 2. Validation status and performance characteristics

### 2.1 General

The method has been optimised for suitable DNA extracted from maize seeds.

The reproducibility and trueness of the method were tested through an international collaborative study using DNA samples at different GMO contents.



## 2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories in December 2006.

Each participant received twenty unknown samples containing MIR604 maize genomic DNA at five GM contents, ranging from 0.1 % to 6.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM MIR604 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found under <http://gmo-crl.jrc.it/statusofdoss.htm>

## 2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is < 0.045% in 250 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

## 2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is < 0.09% in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.1 %.

## 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to MIR604 event and thus imparts event-specificity to the method.

The specificity of the MIR604 assay (forward/reverse oligonucleotide primers and probe) was tested in real-time PCR against DNA extracted from samples containing the specific targets of maize MON810, Bt11, Bt176, GA21, NK603 and MIR604.

None of the above mentioned GM lines tested, except the positive control MIR604, produced amplification signals in replicated samples when 250 ng total DNA per reaction were applied. A relatively weak amplification, with Ct values of 39.76 and 39.04 (average of three samples), was reported with 100% GA21 DNA at 250 ng and 500 ng per reaction (Ct values for reference system of 23.26 and 22.35, respectively). However, no signal was reported when both 50 ng and 1000 ng of GA21 DNA per reaction were used (Ct=40 for MIR604 assay and 22.36 and 21.46 for the reference assay, respectively). In an additional set of experiments, no amplification signal was

obtained with MIR604 assay on GA21 samples (three replicates per concentration level) at 200 and 400 ng per reaction, while the reference assay showed amplifications in the expected range.

### **3. Procedure**

#### **3.1 General instructions and precautions**

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. "flow direction" during PCR-setup, should follow the guidelines given by relevant authorities as e.g. ISO, CEN, Codex Alimentarius Commission.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

## 3.2 Real-time PCR for quantitative analysis of MIR604 maize

### 3.2.1 General

The PCR set-up for the taxon specific target sequence (*Adh1*) and for the GMO (MIR604) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 250 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

### 3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of MIR604 DNA in a total amount of 250 ng maize DNA. The GM content of the standard samples ranges from 10.0% to 0.1%.

A calibration curve is produced by plotting the  $\Delta C_t$ -values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve ( $y = ax + b$ ) are then used to calculate the mean % GM content of the blind samples based on their normalised  $\Delta C_t$  values.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents on ice.**
2. In two reaction tubes (one for MIR604 system and one for the *Adh1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

**Table 1.** Amplification reaction mixture in the final volume/concentration per reaction well for the maize *Adh1* reference system.

Component	Final concentration	µl/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
50x Zm <i>Adh1</i> Endogenous Assay stock	1x	0.5
Nuclease free water	#	7
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

**Table 2.** Amplification reaction mixture in the final volume/concentration per reaction well for the MIR604 specific system.

Component	Final concentration	µl/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
50x Event MIR604 Assay Stock	1x	0.5
Nuclease free water	#	7
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the MIR604 and one for the *Adh1* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g.  $20 \times 3 = 60$  µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g.  $5 \times 3 = 15$  µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
- Spin down the tubes in a microcentrifuge. Aliquot 25 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately  $250 \times g$  for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR with cycling conditions described in Table 3:

**Table 3.** Cycling program for maize MIR604/*Adh1* systems

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	UNG	50 °C	120	No	1
2	Initial denaturation	95 °C	600	No	1
3	Amplification	Denaturation	95 °C	No	40
		Annealing & Extension	60 °C	Yes	

### 3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. MIR604) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at  $Ct = 25 - 3 = 22$ ).
- c) Save the settings.
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *Adh1* system).
- e) Save the settings and export all the data into an Excel file for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the Ct-values for each reaction.

The Reference  $\Delta C_t$ -curve is generated by plotting the  $\Delta C_t$ -values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the regression formula is used to estimate the relative amount (%) of MIR604 event in the unknown samples of DNA.

## 4. Materials

### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

### 4.2 Reagents and solutions

- Sigma Jumpstart RedTaq PCR master mix (2X). Sigma Aldrich Ltd P-2893
- Sulforhodamine 101, Sigma Cat No S-7635
- 50x Zm Adh1 Endogenous Assay Stock:
  - For 1ml of 50x Zm Adh1 Endogenous Assay Stock:
    - 15 µl of Zm Adh1 primer F (1000 pmol/µl)
    - 15 µl of Zm Adh1 primer R (1000 pmol/µl)
    - 100 µl of Zm Adh1 probe (100 pmol/µl)
    - 870 µl of nuclease-free water

The 1x Zm Adh1 Endogenous Assay Stock contains: 300nM Zm Adh1 primer F, 300nM Zm Adh1 primer R and 200nM Zm Adh1 probe.  
Vortex well and store at 4°C for up to 1 year.

- 50x Event *MIR604* Assay Stock:
  - For 1ml of 50x Event *MIR604* Assay Stock:
    - 30 µl of *MIR604* primer F (1000 pmol/µl)
    - 15 µl of *MIR604* primer R (1000 pmol/µl)
    - 100 µl of *MIR604* probe (100 pmol/µl)
    - 855 µl of nuclease-free water

The 1x Event MIR604 Assay Stock contains: 600 nM MIR 604 primer F, 300 nM MIR604 primer R and 200 nM MIR604 probe.

Vortex well and store at 4°C for up to 1 year.

- 10000x Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 ml nuclease free water to make a 1.5 mM stock solution.

Vortex well and store at -20°C.

- Sigma Jumpstart ReadyMix 2x:

➤ For 50 ml: to Sigma Jumpstart RedTaq PCR master mix (2X), add:

- 550 µl of 1M MgCl<sub>2</sub>
- 10 µl 10000x Sulforhodamine 101

Vortex well and store at 4°C for up to 1 year.

### 4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>MIR604 target sequence</i>	
MIR604 primer F	5' –GCG CAC GCA ATT CAA CAG-3'
MIR604 primer R	5' –GGT CAT AAC GTG ACT CCC TTA ATT CT-3'
MIR604 probe	FAM 5'- AGG CGG GAA ACG ACA ATC TGA TCA TG-3' TAMRA
<i>Reference gene Adh1 target sequence</i>	
Zm <i>Adh1</i> primer F	5' –CGT CGT TTC CCA TCT CTT CCT CC-3'
Zm <i>Adh1</i> primer R	5' –CCA CTC CGA GAC CCT CAG TC -3'
Zm <i>Adh1</i> (Probe)	VIC 5' –AAT CAG GGC TCA TTT TCT CGC TCC TCA-3' TAMRA



# **Event-specific Method for the Quantification of Maize Line GA21 Using Real-time PCR**

## **Protocol**

**30 March 2010**

**Joint Research Centre  
Institute for Health and Consumer Protection  
Molecular Biology and Genomics Unit**

### **Method development:**

Syngenta Seeds S.A.S.

### **Collaborative trial:**

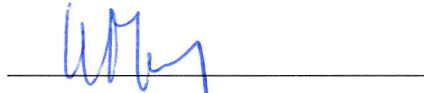
Community Reference Laboratory for GM Food and Feed (CRL-GMFF)  
Molecular Biology and Genomics Unit



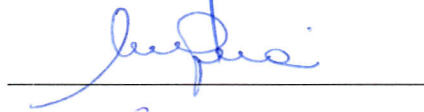
*Drafted by*  
S. Larcher



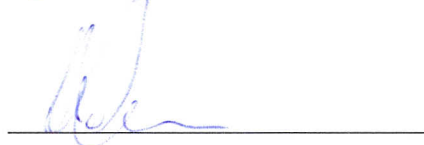
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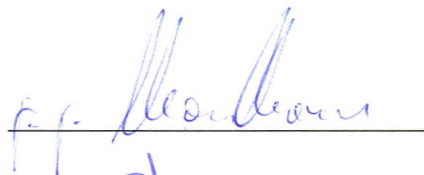
2) M. Querci



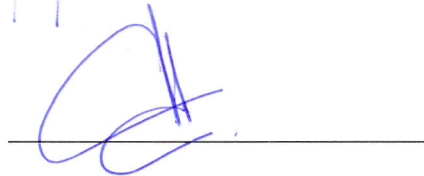
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## 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event GA21 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in the PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of event GA21 DNA, a 101-bp fragment of the recombination region between the insert and the plant genome (located at the 5' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For the relative quantification of event GA21 DNA, a maize-specific reference system amplifies a ~~136-bp~~ 135-bp fragment of the maize endogenous *Alcohol dehydrogenase* gene (*Adh1*), using a pair of specific primers and an *Adh1* gene-specific probe labelled with VIC and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event GA21 DNA in a test sample, the normalised  $\Delta C_t$  values of the calibration samples are used to calculate by linear regression a reference curve  $\Delta C_t$ -formula. The normalised  $\Delta C_t$  values of the unknown samples are measured and, by means of the regression formula, the relative amount of GA21 event DNA is estimated.

## 2. Validation status and performance characteristics

### 2.1 General

The method has been optimised for suitable DNA extracted from maize seeds containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative study using DNA samples at different GM% contents.

## 2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 13 laboratories in June 2006.

Each participant received twenty unknown samples containing GA21 maize genomic DNA at five GM% contents, ranging from 0.09 % to 8.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM GA21 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>

## 2.3 Limit of detection (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.04% in 250 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

## 2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.04% in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09 %.

## 2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to GA21 event and thus imparts event-specificity to the method.

The specificity of the GA21 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR against DNA extracted from samples containing maize MON810, Bt11, Bt176, NK603, GA21 and negative control line for GA21.

None of the above mentioned GM lines tested, except the positive control GA21, produced amplification signals in replicated samples when 250 ng total DNA per reaction were used.

### 3. Procedure

#### 3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. “flow direction” during PCR-setup, should follow the guidelines given by relevant authorities as e.g. ISO, CEN, Codex Alimentarius Commission.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

#### 3.2 Real-time PCR for quantitative analysis of GA21 maize

##### 3.2.1 General

The PCR set-up for the taxon specific target sequence (*Adh1*) and for the GMO (GA21) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 250 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

### 3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of GA21 DNA in a total amount of 250 ng maize DNA. The GM content of the standard samples ranges from 10.0% to 0.09%.

A calibration curve is produced by plotting the  $\Delta C_t$ -values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve ( $y = ax + b$ ) are then used to calculate the mean % GM content of the blind samples based on their normalised  $\Delta C_t$  values.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents on ice.**
2. In two reaction tubes (one for GA21 system and one for the *Adh1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *Adh1* reference system.

Component	Final concentration	µl/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
50x Zm <i>Adh1</i> Endogenous Assay stock	1x	0.5
Nuclease free water	#	7
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the GA21 specific system.

Component	Final concentration	µl/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
50x Event GA21 Assay Stock	1x	0.5
Nuclease free water	#	7
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the GA21 and one for the *Adh1* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g.  $20 \times 3 = 60$  µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g.  $5 \times 3 = 15$  µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
- Spin down the tubes in a microcentrifuge. Aliquot 25 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately  $250 \times g$  for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR with cycling conditions described in Table 3

Table 3. Cycling program for maize GA21/*Adh1* systems

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	UNG	50 °C	120	No	1
2	Initial denaturation	95 °C	600	No	1
3	Amplification	Denaturation	95 °C	No	40
		Annealing & Extension	60 °C	Yes	

### 3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one system (e.g. GA21) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update (or apply)” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at  $Ct = 25 - 3 = 22$ ).
- c) Save the settings.
- d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *Adh1* system).
- e) Save the settings and export all the data into an Excel file for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the Ct-values for each reaction.

The Reference  $\Delta Ct$ -curve is generated by plotting the  $\Delta Ct$ -values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the regression formula is used to estimate the relative amount (%) of GA21 event in the unknown samples of DNA.

## 4. Materials

### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)



- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

## 4.2 Reagents and solutions

- Sigma Jumpstart Taq ReadyMix (2X). Sigma Aldrich Ltd P-2893
- Sulforhodamine 101, Sigma Cat No S-7635
- 50x Zm Adh1 Endogenous Assay Stock:

- For 1ml of 50x Zm Adh1 Endogenous Assay Stock:
  - 15 µl of Zm Adh1 primer F (1000 pmol/µl)
  - 15 µl of Zm Adh1 primer R (1000 pmol/µl)
  - 100 µl of Zm Adh1 probe (100 pmol/µl)
  - 870 µl of nuclease-free water

The 1x Zm Adh1 Endogenous Assay Stock contains: 300 nM Zm Adh1 primer F, 300 nM Zm Adh1 primer R and 200 nM Zm Adh1 probe.

Vortex well and store at 4 °C for up to 1 year.

- 50x Event GA21 Assay Stock:

- For 1ml of 50x Event GA21 Assay Stock:
  - 45 µl of esGA21-5' forward primer (1000 pmol/µl)
  - 45 µl of esGA21-5' reverse primer (1000 pmol/µl)
  - 100 µl of esGA21-5' probe (100 pmol/µl)
  - 810 µl of nuclease-free water

The 1x Event GA21 Assay Stock contains: 900 nM GA21 primer F, 900 nM GA21 primer R and 200 nM GA21 probe.

Vortex well and store at 4 °C for up to 1 year.

- 10000x Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 ml nuclease free water to make a 1.5 mM stock solution.

Vortex well and store at -20 °C.

- Sigma Jumpstart ReadyMix 2x:

- For 50 ml: to Sigma Jumpstart Taq ReadyMix (2X), add:
  - 550 µl of 1 M MgCl<sub>2</sub>
  - 10 µl 10000x Sulforhodamine 101

Vortex well and store at 4 °C for up to 1 year.

### 4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>GA21 target sequence</i>	
esGA21-5' forward primer	5' – CGT TAT GCT ATT TGC AAC TTT AGA ACA-3'
esGA21-5' reverse primer	5' – GCG ATC CTC CTC GCG TT-3'
esGA21-5' probe	FAM 5'- TTT CTC AAC AGC AGG TGG GTC CGG GT-3' TAMRA
<i>Reference gene Adh1 target sequence</i>	
Zm <i>Adh1</i> primer F	5' – CGT CGT TTC CCA TCT CTT CCT CC-3'
Zm <i>Adh1</i> primer R	5' – CCA CTC CGA GAC CCT CAG TC -3'
Zm <i>Adh1</i> probe	VIC 5' – AAT CAG GGC TCA TTT TCT CGC TCC TCA-3' TAMRA

**EUR 24715 EN– Joint Research Centre – Institute for Health and Consumer Protection**

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**Abstract**

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out a verification study to assess the performance of three quantitative event-specific methods on the maize event Bt11 x MIR604 x GA21 (unique identifier SYN BTØ11-1 x SYN-IR6Ø4-5 x MON-ØØØ21-9) which combines the Bt11, MIR604 and GA21 transformation events. The three methods have been validated individually on single trait events, to detect and quantify each event in maize samples. This study was conducted according to internationally accepted guidelines (1, 2). In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Syngenta Seeds S.A.S. provided the detection methods and the control samples: genomic DNA extracted from homogenised seeds of Bt11 x MIR604 x GA21 maize (NP2673GA21xNP2171Bt11+MIR604), genomic DNA extracted from homogenised seeds of non-GM maize (NP2673/NP2171) and flour ground from seeds of NP2673GA21xNP2171Bt11+MIR604 and from seeds of NP2673/NP2171. The EURL-GMFF prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to the European Network of GMO Laboratories (ENGL) method performance requirements (<http://gmocrl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this EURL-GMFF in-house verification study are made publicly available at

<http://gmo-crl.jrc.ec.europa.eu/>

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